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Reduced Proteolysis of Secreted Gelatin and Yps1-Mediated α-Factor Leader Processing in a *Pichia pastoris kex2* Disruptant

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Heterologous proteins secreted by yeast and fungal expression hosts are occasionally degraded at basic amino acids. We cloned *Pichia pastoris* homologs of the *Saccharomyces cerevisiae* basic residue-specific endoproteases Kex2 and Yps1 to evaluate their involvement in the degradation of a secreted mammalian gelatin. Disruption of the *P. pastoris KEX2* gene prevented proteolysis of the foreign protein at specific monoarginylic sites. The *S. cerevisiae* α-factor preproleader used to direct high-level gelatin secretion was correctly processed at its dibasic site in the absence of the prototypical proprotein convertase Kex2. Disruption of the *YPS1* gene had no effect on gelatin degradation or processing of the α-factor propeptide. When both the *KEX2* and *YPS1* genes were disrupted, correct precursor maturation no longer occurred. The different substrate specificities of both proteases and their mutual redundancy for propeptide processing indicate that *P. pastoris kex2* and *yps1* single-gene disruptants can be used for the α-factor leader-directed secretion of heterologous proteins otherwise degraded at basic residues.

Targeting heterologous proteins for secretion can be an advantageous production strategy with eukaryotic microorganisms. Protein secretion allows for folding and posttranslational modification of the foreign protein and can serve as a means to circumvent toxicity problems associated with the intracellular accumulation of some proteins. Furthermore, downstream processing is usually achieved more easily with secreted proteins than with intracellular proteins.

In *Saccharomyces cerevisiae*, the preproregion of the yeast's α-factor mating pheromone is commonly fused to heterologous proteins to direct their efficient secretion (11). The α-factor propeptide is removed in a late Golgi compartment through cleavage C-terminal to Lys-Arg by the membrane-bound protease Kex2 (27). This prototypical proprotein convertase (also known as kexin; EC 3.4.21.61) is a member of the subtilisin superfamily of serine proteases and is specific for (mostly paired) basic amino acids.

The preprosequence of the *S. cerevisiae* α-factor is also widely used for the secretion of heterologous proteins by the methylotrophic yeast *Pichia pastoris* and is the most successful leader sequence employed thus far in this expression system (15). It is generally assumed that α-factor propeptide processing in *P. pastoris* is also effectuated by a Kex2 homolog. We have used the α-factor preprosequence in *P. pastoris* to obtain efficient secretion of mammalian gelatins with yields as high as ~15 g/liter of clarified fermentation broth (46). These gelatins are nonhydroxylated segments of collagen α-chains, which are essentially in the random coil conformation and, therefore, nongelling (46). Traditional animal gelatin is a versatile biomaterial with both medical and technical applications. Gelatins produced in microbial systems provide the added benefit of being well defined, free of animal-derived contagious agents, and suitable for modification for specific needs (35, 46, 47).

Although gelatin production in *P. pastoris* was very successful in terms of productivity, a 53-kDa gelatin derived from the murine type I collagen α-chain (referred to as Col1a1-2) was proteolytically cleaved C-terminal to two occurrences of the monobasic sequence Met-Gly-Pro-Arg (46). This motif is similar to known nondibasic Kex2 substrates, which led to the hypothesis that the putative *P. pastoris* α-factor leader processing Kex2 homolog was responsible for the observed cleavage. Removal of the Met-Gly-Pro-Arg motifs from the gene construct by site-directed mutagenesis resulted in the production of a predominantly full-length product and a number of shorter fragments present in lower quantities (46). A custom-designed gelatin with no Arg residues was produced completely intact, so we hypothesized that the minor fragments may have originated from limited Kex2-like proteolysis at suboptimal monoarginylic motifs (47). In addition to a Kex2 homolog, the aspartic yapsin proteases (18, 30, 36) could also be involved, since their substrate specificities overlap those of Kex2 in *S. cerevisiae* (10). Yps1 (yapsin 1, previously termed Yap3; EC 3.4.23.41) is the best-characterized yapsin and is primarily active at the plasma membrane but is also found in the extracellular medium and transiently active in the late secretory pathway (2, 14, 28). It has frequently been implicated in the degradation of secreted heterologous proteins at basic amino acids in *S. cerevisiae* (see, for example, reference 28).

Our objective in this study was to isolate and disrupt the *P. pastoris* genes encoding Kex2 and Yps1 and to evaluate the utility of these gene disruptants for the production of intact Col1a1-2 gelatin. The development of *P. pastoris* strains deficient in proteolysis at basic residues should be valuable for the industrial production of medically or technically important proteins.

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MATERIALS AND METHODS

***P. pastoris* strains and media.** The *P. pastoris* GS115-based strain subjected to gene disruptions in this study was the previously described Col1a1-2 gelatin-

producing strain COL1A1-2 (46). The expression vector used in this strain was derived from pPIC9 (Invitrogen, Breda, The Netherlands) and encodes a translational fusion of the *S. cerevisiae* α-factor prepropeptide and 53 kDa of the helical domain of murine type I collagen (32). Strain COL1A1-2* produces a variant of Col1a1-2 referred to as Col1a1-2*, in which the proteolyzed Met-Gly-Pro-Arg motifs had been removed by site-directed mutagenesis of the encoding gene (46).

Yeast extract-peptone-dextrose (YPD) medium (Duchefa, Haarlem, The Netherlands) contained 10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter dextrose. Minimal dextrose medium consisted of 13.4 g/liter yeast nitrogen base without amino acids (Sigma, Zwijndrecht, The Netherlands), 10 g/liter dextrose, and 0.4 mg/liter biotin. Basal salts medium was as described by Zhang et al. (49).

Protein sequence analyses. Pairwise alignment of amino acid sequences and calculation of percent identity and similarity were performed with the EMBOSS Needle program (38). The PAM250 similarity matrix was used, the gap opening penalty was 10, and the gap extension penalty was 0.5. The Pfam server (6) was used to search for functional protein domains. Secretory signal prediction was done with the SignalP neural network (34). Sequences were analyzed for the presence of potential transmembrane domains with SOSUI (25). Glycosylphosphatidylinositol modification sites were predicted with the fungal big-PI predictor (19).

Cloning of *P. pastoris KEX2*. An ~0.4-kb fragment was amplified from *P. pastoris* GS115 genomic DNA with degenerate primers (forward, 5′-CAYGGN ACNMGNTGYGC-3′; reverse, 5′-GTRTANCCRTCRSARTTRCA-3′). The PCR product was cloned into pGEM-T Easy (Promega, Leiden, The Netherlands) and sequenced to confirm its similarity to the *S. cerevisiae KEX2* gene. Inverse PCR (44) was then performed on EcoRI-digested and self-ligated genomic DNA with the Expand Long Template PCR System (Roche, Almere, The Netherlands). Subfragments of the ~5.4-kb inverse PCR product were sequenced, and the entire genomic fragment was subsequently amplified with the Expand High-Fidelity PCR system (Roche). The PCR product was cloned into pGEM-T Easy (Promega), and both strands were sequenced by primer walking (Eurogentec, Seraing, Belgium).

***KEX2* gene disruption.** Plasmid pMTL23-KEX2 was constructed by insertion of an ~3.9-kb BamHI *KEX2* subfragment into the BamHI site of cloning vector pMTL23 (16). The plasmid was cut with Van91I and PinAI, which removed ~1.7-kb of the *KEX2* fragment. An ~1.2-kb Zeocin resistance cassette of vector pPICZα A (Invitrogen) was amplified with primers that introduce a Van91I site at the 5′ end and a PinAI site at the 3′ end (forward, 5′-CTTCCACAATGTG GCCCACACACCATAGCTTC-3′; reverse, 5′-CTGACCGGTAGCTTGCAAA TTAAAGCCTTC-3′). The PCR product was cloned into the cut pMTL23-KEX2 plasmid. For gene replacement, the resulting disruption vector pKEX2Δ was digested with BamHI and the 3.4-kb fragment obtained was used to transform *P. pastoris*. Transformation was done by electroporation (7) in 0.2-cm cuvettes with a Gene Pulser (Bio-Rad, Veenendaal, The Netherlands) set at 1,500 V, 25 μF, and 200 Ω. The electroporated cells were allowed to recover for 1 h in 1 M sorbitol at 30°C. One volume of YPD medium was added, and the cells were grown for 1 h at 30°C before being plated on YPD medium supplemented with 1 M sorbitol and 100 μg/ml Zeocin (Invitrogen).

Preparation of membrane extracts for the Kex2 assay. Overnight cultures of *P. pastoris* were grown in 25 ml of minimal dextrose medium. Cells were harvested by centrifugation at 4°C for 5 min at 1,500 × *g* and washed three times with 25 ml of 100 mM Tris-HCl (pH 7.0) by repeated resuspension and centrifugation. The final cell pellet was resuspended in 1 ml of the same buffer. Acid-washed 425- to 600-μm glass beads (Sigma) were added to a final volume of ~1.8 ml, and the cells were lysed at 4°C in a mixer mill (Retsch, Haan, Germany) for 5 min at 30 Hz. The glass beads were allowed to settle, and the supernatant was spun twice for 5 min at 1,500 × *g* and 4°C to remove any remaining intact cells. The supernatant was centrifuged at 4°C for 2 h at 20,800 × *g* (rotor *k*-factor, 377), and then the pellet was resuspended in 1 ml of 100 mM Tris-HCl (pH 7.0), 0.5 M NaCl and centrifuged as before. This washing step was repeated with 1 ml of 100 mM Tris-HCl (pH 7.0). The resulting membrane pellet was resuspended in 50 μl of 100 mM Tris-HCl (pH 7.0), 1% (vol/vol) Triton X-100. The sample was incubated at 4°C for 30 min with gentle agitation and then centrifuged for 2 h at 20,800 × *g* and 4°C. The protein content of the supernatant was determined with the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as a standard.

Kex2 assay. A modification of the procedure of Achstetter and Wolf (1) was used. An aliquot of 50 μg of the detergent-solubilized membrane proteins (see above) was incubated in 200 μl of 100 mM Tris-HCl (pH 7.0), 0.1% (vol/vol) Triton X-100, 0.5 mM benzyloxycarbonyl-Tyr-Lys-Arg-*p*-nitroanilide (Bachem, Weil am Rhein, Germany), and 5 mM calcium chloride. The release of *p*-nitroaniline at 30°C was monitored for 16 h by measuring the A_{405} in a Safire microplate spectrophotometer (Tecan, Giessen, The Netherlands). The initial velocity was calculated from the slope of the enzyme progress curve over a period of 2.5 h (16 data points). Enzyme activity was calculated using a molar absorption coefficient at 405 nm of 9,500 M^{-1} cm^{-1} for *p*-nitroaniline (21).

Cloning of *P. pastoris YPS1*. An ~0.4-kb fragment was amplified from *P. pastoris* GS115 genomic DNA with degenerate primers (forward, 5′-GCAGGC CACCGTTGACTGCTCGCARTAYGGNACNTT-3′; reverse, 5′-GGTATACT TGCTTTGGTCCACTGCTCCRAANARDAT-3′). The PCR product was cloned with the TOPO TA cloning kit (Invitrogen) and sequenced to verify its similarity to the *S. cerevisiae YPS1* gene. The GeneRacer system (Invitrogen) was used according to the manufacturer's recommendations to isolate the unknown 5′ and 3′ termini of the transcript by RNA ligase-mediated rapid amplification of cDNA ends (33, 45). Several cloned PCR products were sequenced, and the entire transcript was subsequently amplified by reverse transcription-PCR with the Expand High Fidelity PCR System (Roche). The ~1.9-kb fragment obtained was cloned with the TOPO TA cloning kit (Invitrogen), and both strands were sequenced by primer walking (BaseClear, Leiden, The Netherlands).

***YPS1* gene disruption.** The ~0.4-kb *YPS1* fragment isolated by degenerate PCR was reamplified with primers that introduce a BglII site at the 5′ end and a BamHI site at the 3′ end (forward, 5′-GCGAGATCTGCAGGCCACCGTT GACTG-3′; reverse, 5′-GCAGGATCCGGTATACTTGCTTTGGTCCAC-3′). An ~2.0-kb fragment containing a blasticidin resistance cassette and an *Escherichia coli* origin of replication was cut from vector pPIC6 A (Invitrogen) with BglII/BamHI. The PCR product was digested with BglII/BamHI and ligated into the vector fragment. Of the two possible orientations, a clone was chosen in which the fragments were oriented such that it had nonfunctional hybrid BglII/BamHI sites. For insertional gene disruption, the vector pYPS1Δ so obtained was linearized within the *YPS1* fragment with NdeI prior to transformation. Transformation of *P. pastoris* was as described for *KEX2* disruption, but selection was with 300 μg/ml blasticidin (Invitrogen).

Yapsin assay. The yapsin assay is modified from Azaryan et al. (4). Membrane extracts were prepared as described for the Kex2 assay, but 100 mM sodium citrate buffer (pH 4.0) was used in all steps instead of 100 mM Tris-HCl (pH 7.0) due to the different pH optima of Kex2 and Yps1. An aliquot of 50 μg of the detergent-solubilized membrane proteins was incubated for 48 h at 30°C in 200 μl of 100 mM sodium citrate buffer (pH 4.0), 0.1% (vol/vol) Triton X-100, 0.5 mM benzyloxycarbonyl-Tyr-Lys-Arg-*p*-nitroanilide (Bachem), and 5 mM calcium chloride. Subsequent treatment with aminopeptidase M was required to release *p*-nitroaniline after the initial Yps1 reaction. This treatment is consistent with the reported need for aminopeptidase M treatment with fluorogenic methylcoumarin amide peptides, which are cleaved between the paired basic residues by *S. cerevisiae* Yps1 (4), possibly due to the steric constraints imposed by peptide substrates. The reaction mixtures were heat-inactivated by boiling for 5 min, after which 30 μl of 1 M Tris-HCl (pH 9.0) was added to raise the pH to 7.5 to enable aminopeptidase M activity. A 200-μl portion of each sample was transferred to a microtiter plate, and the A_{405} was read in a microplate spectrophotometer (Tecan Safire). The samples were incubated for ~2 h at 37°C after addition of 20 mU of aminopeptidase M (Roche) until the absorbance remained constant. The increase in absorbance after addition of aminopeptidase M was used as a measure of endopeptidase cleavage during the incubation of the membrane extracts with the chromogenic substrate. Enzyme activity was calculated using a molar absorption coefficient at 405 nm of 9,500 M^{-1} cm^{-1} for *p*-nitroaniline (21).

Fermentation of *P. pastoris*. Fed-batch fermentations were performed in 2.5-liter Bioflo 3000 fermenters (New Brunswick Scientific, Nijmegen, The Netherlands), essentially as described by Zhang et al. (49). Minimal basal salts medium was used, and no protease-inhibiting supplements were added. The pH during the glycerol batch phase was kept at 5.0. The pH was allowed to decrease metabolically to 3.0 during the glycerol fed-batch phase and was maintained at this pH during the 3- to 4-day methanol fed-batch phase. A homemade semiconductor gas sensor-controller, similar to that described by Katakura et al. (29), was used to monitor the methanol level in the off-gas and to maintain a constant level of ~0.2% (wt/vol) methanol in the broth.

Incubation experiments for extracellular precursor processing. Fresh cells of the *kex2* disruptant were obtained from fermentation broth harvested just prior to methanol induction, such that the pH was 3.0 but gelatin was not yet being produced. The cells were pelleted by centrifugation for 5 min at 1,500 × *g* and 4°C and washed three times with deionized water by repeated resuspension and centrifugation.

Samples of the *kex2 yps1* disruptant were harvested at the end of the fermentation. Cell-free supernatant was prepared by centrifugation of the broth for 5 min at 1,500 × *g* and 4°C, followed by microfiltration of the supernatant using disposable 0.2-μm cellulose acetate syringe filters (Schleicher & Schuell, 's-Hertogenbosch, The Netherlands).

A 1:1 ratio was used when *kex2 yps1* cell-free supernatant was mixed with *kex2* cell-free supernatant or cell-containing broth. When *kex2* cells were mixed with *kex2 yps1* supernatant, they were resuspended at ~450 mg (wet weight)/ml to mimic a typical high-cell-density fermentation. The aspartic protease inhibitor pepstatin was optionally added at 10 μM. The mixtures were incubated at 30°C for 8 h and microfiltered to remove cells where applicable.

SDS-PAGE and N-terminal protein sequencing. The NuPAGE Novex system (Invitrogen) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 10% Bis-Tris gels, morpholineethanesulfonic acid (MES) SDS running buffer and SeeBlue Plus2 prestained molecular mass markers. Gelatin was purified by differential acetone precipitation (46) prior to electrophoresis. Gels were stained with Coomassie SimplyBlue SafeStain (Invitrogen). Blotting of proteins for N-terminal sequencing by Edman degradation was as described previously (46). Protein sequencing was performed by Midwest Analytical (St. Louis, Mo.).

Nucleotide sequence accession number. The nucleotide sequence data for *KEX2* and *YPS1* have been deposited in the GenBank database under accession no. AY362700 and AY362701, respectively.

RESULTS

Cloning of *P. pastoris KEX2* and characterization of the deduced protein sequence. A putative *P. pastoris KEX2* fragment encoding the catalytic domain was isolated by PCR with degenerate primers based on the amino acid sequences from various yeasts, and the entire gene was isolated by inverse PCR.

Among fungal Kex2 homologs, the deduced *P. pastoris* amino acid sequence is most similar to that of *Kluyveromyces lactis* (EMBL accession no. CAA30088; 45% identity and 66% similarity) and *S. cerevisiae* (GenPept accession no. AAA34718; 44% identity and 66% similarity). The first 21 residues of the *P. pastoris* sequence probably are a signal peptide for translocation into the endoplasmic reticulum. A conserved dibasic site (residues 114 to 115) defines a putative proregion, which in *S. cerevisiae* is autocatalytically processed in the endoplasmic reticulum (22). A region downstream of the supposed propeptide probably is a subtilisin domain (residues 152 to 445) and contains the highly conserved Asp, His, and Ser subtilisin catalytic triad as well as a conserved Asn residue that stabilizes the oxyanion in the transitional state during hydrolysis of the peptide bond (12). The region downstream of the catalytic domain probably is a proprotein convertase P-domain (residues 466 to 598), which in *S. cerevisiae* is required for the intramolecular maturation of pro-Kex2 (23). A Ser/Thr-rich domain, probably a target for O-glycosylation, is found C-terminal to this region, followed by a transmembrane domain. The C-terminal cytoplasmic tail contains a conserved Tyr residue (position 716) that, together with contextual sequences, constitutes a *trans*-Golgi network localization signal in *S. cerevisiae* (48).

Cloning of *P. pastoris YPS1* and characterization of the deduced protein sequence. Degenerate primers were designed on the basis of a yapsin-like gene fragment from *Pichia angusta* (EMBL accession no. AL433341) and conserved amino acid stretches of *S. cerevisiae* Yps1, Yps2, and Yps3 (RefSeq accession no. NP_013221, NP_010428, and NP_013222, respectively). Reverse transcription-PCR with these primers followed by rapid amplification of cDNA ends resulted in the isolation of a putative *P. pastoris* yapsin.

Comparison of the deduced amino acid sequence with the *S. cerevisiae* yapsins showed that it was most similar to Yps1 (41% identity and 65% similarity). The first 23 residues probably are a secretory signal. A dibasic propeptide processing site, which is presumably cleaved autocatalytically in *S. cerevisiae* (14), is conserved in the *P. pastoris* sequence (residues 70 to 71). The location of the two active site Asp residues (residues 104 and 384) and their specific sequence context match those of *S. cerevisiae* Yps1 (3). Two Cys residues are also conserved (residues 120 and 202). In *S. cerevisiae* Yps1, these residues flank an exposed loop region, which is proteolytically cleaved into α- and β-subunits that are linked by a disulfide bridge (14). The inferred loop region in the *P. pastoris* sequence contains a stretch of Gly-Ser repeats that may confer conformational flexibility. The C-terminal part of the sequence contains a Ser/Thr-rich region (residues 545 to 569) that may be O-glycosylated, a hydrophobic tail (residues 579 to 599), and a putative glycosylphosphatidylinositol (GPI) modification signal (19). Most GPI-anchored plasma membrane proteins, including *S. cerevisiae* Yps1, have a pair of basic amino acids just prior to the GPI attachment site (24). The putative GPI attachment site of *P. pastoris* Yps1 (residue 574) is immediately preceded by a triplet of acidic residues. The significance of this atypical sequence context is unclear.

Disruption of the *KEX2* and *YPS1* genes. The *KEX2* and *YPS1* genes were disrupted individually and in combination in the present study, using a previously described *P. pastoris* strain that produces Collal-2 gelatin (46) and which is hereafter referred to as the wild type. The part of the *KEX2* locus encoding the catalytic and P domains was replaced with the Zeocin resistance cassette of plasmid pKEX2Δ (Fig. 1A). The *YPS1* gene was disrupted by insertion of the blasticidin-selectable plasmid pYPS1Δ in the region that encodes the catalytic β-subunit (Fig. 1B), N-terminal to the subunit's catalytic Asp residue. Correct disruption of the *KEX2* and *YPS1* genes was verified by PCR (not shown) and Southern blotting (Fig. 1C and D).

To demonstrate functional disruption of the *KEX2* and *YPS1* genes, the corresponding enzyme activities of all three disruptants and the wild type were assayed (Table 1). Kex2 activity was negligible in the *kex2* and *kex2 yps1* disruptants compared to the wild type and the *yps1* disruptant. A control reaction consisting of a wild-type sample and the chelator EGTA instead of the Ca^{2+} cofactor had minimal Kex2 activity, which attests to the specificity of the assay. Yapsin activity was greatly reduced in the *yps1* and *kex2 yps1* disruptants, demonstrating functional disruption of the *YPS1* gene. A wild-type control reaction performed in the presence of the aspartic protease inhibitor pepstatin appropriately showed little yapsin activity. For unknown reasons, the yapsin activity in the *kex2* single disruptant was increased by 95% relative to the wild type.

Phenotype of protease disruptants. A number of phenotypic defects have been described for *kex2* mutants of different yeast species (5, 17, 20, 30). We did not observe obvious aberrant morphologies of cells grown in YPD, nor did we notice any growth arrest at 16°C as reported for *S. cerevisiae* (30). The generation time of *P. pastoris kex2* and *kex2 yps1* disruptants grown in YPD medium at 30°C was increased by ~50% relative to the wild type (data not shown). The morphology and growth rate of the *yps1* disruptant in YPD were indistinguishable from the wild type. In practical terms, all strains had good viability and were amenable to high-cell-density fermentation (~350 to 550 g [wet weight]/liter).

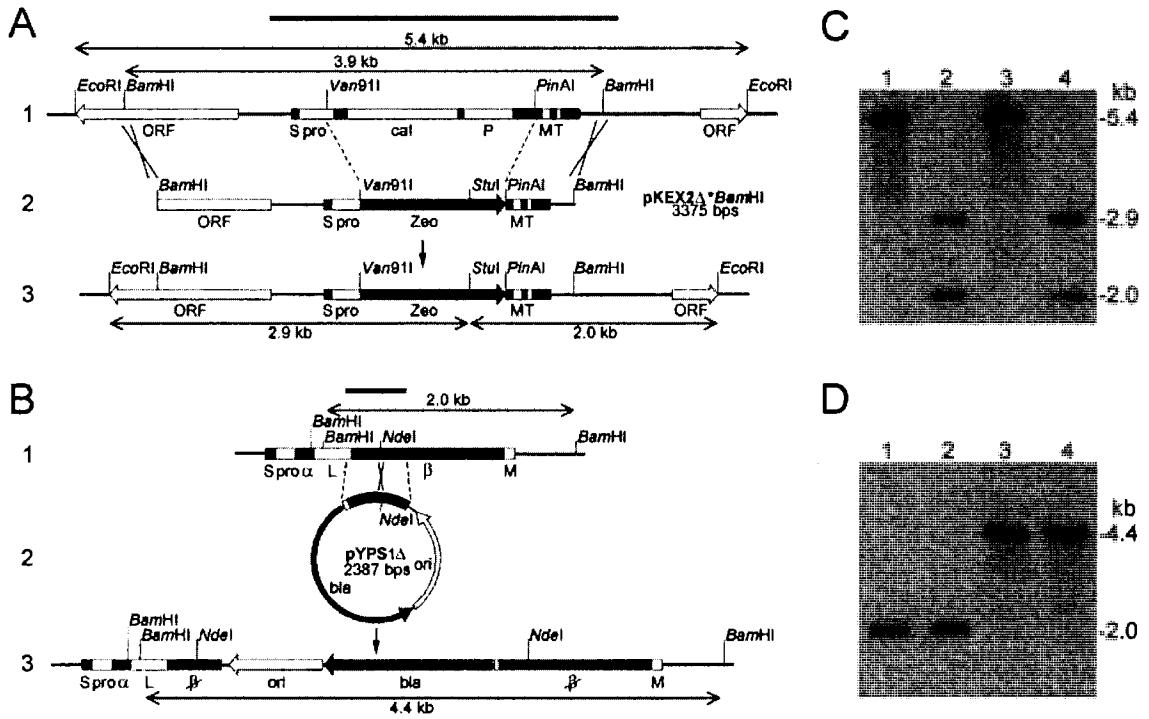

FIG. 1. *KEX2* and *YPS1* gene disruption. (A) Schematic representation of the *P. pastoris KEX2* locus and its disruption. Abbreviations: ORF, unrelated flanking open reading frame; S, signal peptide; pro, propeptide; cat, catalytic domain; P, P domain; M, membrane-spanning domain; T, *trans*-Golgi network localization signal; Zeo, Zeocin resistance cassette of pPICZα A (Invitrogen). The three parts of the scheme represent the *KEX2* locus (1), plasmid pKEX2Δ digested with BamHI (2), and the disrupted *KEX2* locus (3). The black bar indicates the region used as a probe for Southern blotting. (B) Disruption of *P. pastoris YPS1*. Abbreviations: S, signal peptide; pro, propeptide; α, α-subunit; L, loop region; β, β-subunit (strikethrough indicates disruption); M, membrane anchoring GPI domain; bla, blasticidin resistance cassette of pPIC6 A (Invitrogen); ori, *E. coli* origin of replication of pPIC6 A. The three parts of the scheme represent the *YPS1* locus (1), plasmid pYPS1Δ digested with NdeI (2), and the disrupted *YPS1* locus (3). The black bar represents the probe used for Southern blotting. (C) Verification of *KEX2* disruption by Southern blotting. Genomic DNA digested with EcoRI/StuI was hybridized with the *KEX2* probe indicated in panel A. Lanes: 1, wild type; 2, *kex2*; 3, *yps1*; 4, *kex2 yps1*. The hybridization pattern was as expected (5.4 kb for the wild type and the *yps1* disruptant and 2.9 and 2.0 kb for the *kex2* disrupted strains). (D) Verification of *YPS1* disruption by Southern blotting. Genomic DNA digested with BamHI was hybridized with the *YPS1* probe indicated in panel B. The lanes correspond to the same strains as in panel C. The hybridization pattern was as expected (2.0 kb for the wild type and the *kex2* disruptant and 4.4 kb for the *yps1* disruptants).

Gelatin production in protease disruptants. Use of the *kex2* disruptant for the production of Col1a1-2 prevented the predominant proteolysis that occurs in the wild-type strain. While major fragments resulting from cleavage at Met-Gly-Pro-Arg are seen in the wild type (Fig. 2, lane 1, bands 4 to 6), these fragments were absent in the *kex2* disruptant (Fig. 2, lane 3). This shows that Kex2 is responsible for the cleavage observed in the wild type. The banding pattern of the remaining minor degradation products in the *kex2* disruptant was very similar to that of the mutated Col1a1-2* gelatin produced in the wild type (46), which lacks the proteolyzed Met-Gly-Pro-Arg sites (Fig. 2, lane 2). This result indicates that Kex2 is not an important contributor to the formation of these minor fragments.

Col1a1-2 produced by the *yps1* disruptant (Fig. 2, lane 4) had a banding pattern virtually identical to that of the wild type (Fig. 2, lane 1). Except for two diffuse bands (bands 1 and 2) just over the full-length band (band 3), gelatin produced in the *kex2 yps1* double disruptant (Fig. 2, lane 5) was similar to that produced in the *kex2*-disrupted strain (Fig. 2, lane 3). Disruption of the *YPS1* gene apparently has no significant effect on the integrity of Col1a1-2.

TABLE 1. Kex2 and yapsin activities of wild-type and disruptant strains[a]

Strain	Relative activity (%) of:	
	Kex2	Yapsin
Wild type	100 ± 2	100 ± 1
kex2	1 ± 1	200 ± 4
yps1	96 ± 0.4	5 ± 3
kex2 yps1	1.0 ± 0.5	11 ± 3
Wild type[b]	2.4 ± 0.4	6 ± 4

[a] Values are means ± standard deviations (n = 3) and are expressed as percentages of the mean wild-type activities (1.7 and 0.037 nmol/min per mg of solubilized membrane protein for the Kex2 and yapsin assays, respectively).

[b] Control reactions in which the Kex2 assay contained the chelator EGTA instead of the Ca^{2+} cofactor and in which the yapsin assay contained the aspartic protease inhibitor pepstatin. The accessory aminopeptidase M used in the yapsin assay was basically unaffected by pepstatin (not shown).

Characterization of α-factor leader processing. The major Col1a1-2 band produced by the *kex2* disruptant (Fig. 2, lane 3, band 3) migrated at the same position as that of Col1a1-2* produced by the wild type (Fig. 2, lane 2, band 3). As the

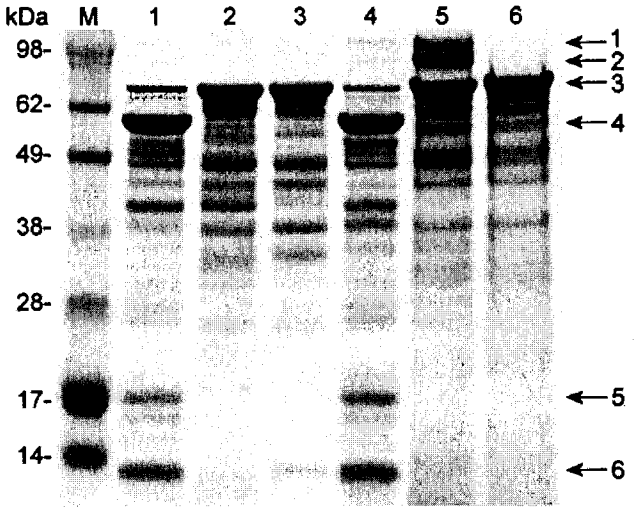

FIG. 2. SDS-PAGE of gelatin produced in wild-type and disruptant strains. Lanes correspond to gelatin type and strain, respectively, as follows: lane 1, Col1a1-2/wild type (46); lane 2, Col1a1-2*/wild type (46); lane 3, Col1a1-2/*kex2*; lane 4, Col1a1-2/*yps1*; lane 5, Col1a1-2/*kex2 yps1*; lane 6, endoglycosidase H-treated Col1a1-2/*kex2 yps1*. Lane M, molecular mass marker. Arrows indicate protein bands referred to in the text. Gelatin was purified from the clarified broth by differential acetone precipitation (46). The equivalent of 5 μl of clarified broth was loaded for all samples, except for lanes 5 and 6, in which the equivalent of 15 μl was loaded for approximate normalization. Note that gelatins migrate in SDS-PAGE at an apparent molecular mass ~40% higher than the true molecular mass (46).

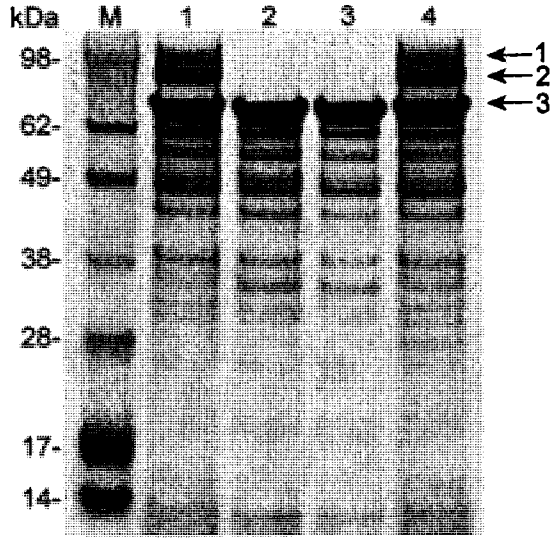

FIG. 3. SDS-PAGE of extracellular precursor processing. Lanes correspond to the following incubations: lane 1, *kex2 yps1* cell-containing broth; lane 2, cell-free *kex2 yps1* supernatant mixed with *kex2* cells; lane 3, cell-free *kex2 yps1* supernatant mixed with *kex2* cell-free supernatant; lane 4, cell-free *kex2 yps1* supernatant mixed with *kex2* cell-containing broth in the presence of pepstatin. Lane M, molecular mass marker. Protein bands are indicated as follows: arrows 1 and 2, Col1a1-2 precursors; arrow 3, mature Col1a1-2. Gelatin was purified by differential acetone precipitation (46). The equivalent of 15 μl of *kex2 yps1* fermentation supernatant was loaded in each lane.

α-factor propeptide of the latter is correctly processed (46) and unprocessed precursors would have had a visibly higher molecular mass in SDS-PAGE, some form of α-factor leader processing must have occurred with the gelatin produced by the *kex2* disruptant. The N-terminal sequence of the major Col1a1-2 band from the *kex2* disruptant (Fig. 2, lane 3, band 3) was Glu-Ala-Glu-Ala-Pro. This corresponds to the sequence immediately C-terminal to the dibasic site in the α-factor propeptide/Col1a1-2 fusion and indeed reveals accurate Kex2-like processing of the propeptide. The sequence also reveals a lack of processing of the α-factor-derived $(Glu\text{-}Ala)_2$ spacer by the late Golgi protease dipeptidyl aminopeptidase A (DPAPase A) (26).

The N-terminal sequence of the full-length band obtained with the *yps1* disruptant (Fig. 2, lane 4, band 3) was Glu-Ala-Pro-Met-Gly, which is identical to the sequences previously reported for the full-length bands of Col1a1-2 and Col1a1-2* produced in the wild type (46). The sequence reveals correct cleavage at the dibasic site of the α-factor propeptide and partial DPAPase A processing of the $(Glu\text{-}Ala)_2$ spacer (i.e., removal of one of the two Glu-Ala repeats).

The two diffuse bands appearing in the *kex2 yps1* double disruptant (Fig. 2, lane 5, bands 1 and 2) disappeared following treatment of the protein with endoglycosidase H (Fig. 2, lane 6). As Col1a1-2 produced in the wild type is not glycosylated (not shown), the effect of endoglycosidase H probably reflects the removal of oligosaccharides from small remnants of the α-factor propeptide, which is known to be glycosylated. N-terminal sequencing of the main and the diffuse (glycosylated) bands from the *kex2 yps1* disruptant (Fig. 2, lane 5, bands 1 to 3) confirmed the presence of such remnants. The following sequences internal to the α-factor propeptide were found: band 1, Asp-Val-Ala-Val-Leu and Val-Ala-Val-Leu-Pro; band 2, Phe-Ile-Xaa-Thr-Thr; band 3, Glu-Glu-Gly-Val-Ser and Glu-Lys-Arg-Glu-Ala. Apparently, α-factor propeptide cleavage at the dibasic site does not occur in the *kex2 yps1* double disruptant, and the aberrant precursor maturation seen is probably caused by other proteases. This shows that Yps1 mediated the α-factor propeptide cleavage at the dibasic site observed in the *kex2* single disruptant.

Extracellular precursor processing. Yps1 activity in *S. cerevisiae* is detectable at the cell surface (31) and in the extracellular medium (2, 28), so extracellular Kex2-independent precursor processing in fermentations of *P. pastoris* was evaluated (Fig. 3) by mixing cell-free supernatant of the *kex2 yps1* double disruptant (containing incompletely processed gelatin precursors) with either washed *kex2* cells or cell-free *kex2* supernatant (harvested prior to induction of gelatin expression). The gelatin precursors produced by the *kex2 yps1* double disruptant appeared to be processed when incubated with washed *kex2* cells (Fig. 3, lane 2) and when incubated with cell-free *kex2* supernatant (Fig. 3, lane 3) but not in control incubations. The control incubations contained either *kex2 yps1* cell-containing broth alone (Fig. 3, lane 1) or a mixture of cell-free *kex2 yps1* supernatant with cell-containing *kex2* fermentation broth in the presence of the aspartic protease inhibitor pepstatin (Fig. 3, lane 4).

DISCUSSION

We cloned *P. pastoris* homologs of the *S. cerevisiae* Kex2 and Yps1 basic residue-specific endoproteases. Both deduced protein sequences were highly similar to the *S. cerevisiae* prototypes and contained all of the expected functional domains. Disruption of the putative *P. pastoris KEX2* and *YPS1* genes caused loss of most activity in corresponding enzyme assays, which confirms the identity of the genes. The near lack of yapsin activity in the *yps1* disruptant also suggests that Yps1 represents the major yapsin in *P. pastoris*, at least under the conditions used. The measured yapsin activity of the *kex2* disruptant was ~95% higher than that of the wild-type strain. Yps1 is clearly the major contributor to this increased activity, given the low activity of the *kex2 yps1* disruptant. Accordingly, a preliminary assay showed that ~90% of the activity in the *kex2* disruptant was inhibited by the aspartic protease inhibitor pepstatin. We do not know whether the increased yapsin activity in the *kex2* disruptant is the result of a regulated, compensatory response to the lack of Kex2 activity.

Production of Col1a1-2 gelatin in the *kex2* disruptant prevented the prominent cleavage normally observed at the Met-Gly-Pro-Arg motifs, which confirms our prior hypothesis that Kex2 was responsible for this monobasic cleavage (46). The banding pattern of low-level degradation products in the *kex2* strain was not significantly different from that in the wild type. We previously suggested that these minor fragments might have originated from limited cleavage by Kex2 at Arg residues within a suboptimal sequence context because a tailored gelatin with no Arg residues at all was secreted completely intact (47) and because even the relatively discriminative S_2 and S_4 subsites (Schechter and Berger [42] nomenclature) of *S. cerevisiae* Kex2 have some leeway in the substrate residues that they can accommodate (8, 43). Apparently, however, few sites in Col1a1-2 other than Met-Gly-Pro-Arg meet the Kex2 protease's overall substrate requirements, as most of the minor bands were also present in the *kex2* disruptant.

The integrity of Col1a1-2 produced by the *yps1* disruptant was no better than that of gelatin produced by the wild type. The degradation seen in the *kex2 yps1* double disruptant was similar to that in the *kex2* disruptant. Thus, Yps1 does not seem to be an important contributor to the degradation of Col1a1-2. Yps1 was involved in the Kex2-independent processing of the α-factor propeptide, as the Col1a1-2 precursor was properly processed in a *kex2* disruptant but not in the *kex2 yps1* double disruptant. Col1a1-2 is secreted at 2 to 3 g/liter in wild-type *P. pastoris* (46), and the productivity of the *kex2* disruptant as judged by SDS-PAGE is similar (Fig. 2). It was unexpected that a *P. pastoris kex2* disruptant can completely mature gelatin secreted at such high levels, since the *S. cerevisiae KEX2* gene was first isolated by complementation of *kex2* mutants that were sterile due to their inability to produce mature α-factor (27). Furthermore, maturation of α-factor/somatostatin precursors did not occur in a *kex2* mutant of *S. cerevisiae* (9). Through the use of a sensitive halo assay, Egel-Mitani et al. (18) demonstrated production of mature α-factor by an *S. cerevisiae kex2* mutant when either Yps1 or the prepropheromone itself was overexpressed. This result suggests that only a portion of the α-factor molecules were processed in this mutant, which is consistent with the partial processing reported for an α-factor/insulin hybrid in *S. cerevisiae* (18). In comparison, the precursor processing observed during the fermentation of the *P. pastoris kex2* disruptant appears rather efficient.

While Kex2 is thought to recognize only the region N-terminal to the cleaved bond (9, 40), *S. cerevisiae* Yps1 is predicted to interact with at least three residues on the C-terminal side (37). The particular protein fused to the α-factor propeptide may therefore influence precursor processing, and we cannot formally exclude the possibility that the Col1a1-2 precursor is more efficiently processed than other α-factor propeptide fusions. However, the Col1a1-2 precursor is not expected to be a uniquely well-suited substrate for Yps1, as it lacks the basic amino acids that Yps1 is proposed to favor among the first three (37) or six (13) residues C-terminal to the cleavage site.

S. cerevisiae Yps1 is localized to the extracellular side of the plasma membrane via its GPI anchor. It may play a role in the proteolytic activation of secreted enzymes or the shedding of membrane receptors (2). Before reaching the periplasm, Yps1 may be transiently active during its passage through the late secretory pathway (2, 14). This hypothesis is supported by the secretion of biologically active α-factor by an *S. cerevisiae kex2* mutant (18) because the pheromone is active only when its N-terminal Glu-Ala repeats are removed by the *trans*-Golgi protease DPAPase A (26), following cleavage at Lys-Arg. In wild-type *P. pastoris*, only one of the two Glu-Ala repeats of Col1a1-2 was processed (46), probably due to steric hindrance by the adjacent Pro residue. In the *kex2* disruptant, however, neither of the two Glu-Ala repeats was processed by DPAPase A. The high level of gelatin secreted may have exceeded the capacity of any transient Yps1, and the prerequisite dibasic cleavage of the α-factor propeptide might not have occurred until the gelatin was beyond the Golgi and its DPAPase A.

Yapsin activity is detectable at the cell surface of *S. cerevisiae* (31) and Yps1 is also present in the extracellular medium (2, 28). The release of GPI-anchored Yps1 from the cell may be due to specific phospholipases (2). In high-cell-density fermentations, at least some of the Yps1 released probably is due to cell lysis. To determine whether *P. pastoris* Yps1 can process the α-factor leader in the fermentation broth, cell-free supernatant of the *kex2 yps1* disruptant was incubated with washed *kex2* cells or with cell-free *kex2* supernatant. In both cases, the precursor forms produced by the *kex2 yps1* double disruptant are processed, suggesting the presence of both cell surface-associated and free Yps1 activity. Be it cell associated or free, Yps1 activity is present in the fermentation broth at pH 3.0. Extracellular Yps1 probably is generally active in *P. pastoris* fermentations, as the pH range of 3.0 to 5.0 common for *P. pastoris* fermentations approximates the active pH range for *S. cerevisiae* Yps1 (4). Yps1 activity in the broth may contribute to the efficient α-factor propeptide processing observed in the *kex2* disruptant, although the precursor molecules could already have been processed by Yps1 in the periplasmic space.

The *S. cerevisiae* Kex2 and Yps1 proteases have overlapping but distinct substrate specificities (10). For example, the S_1 subsite of Kex2 is highly specific for Arg (39, 41), while Yps1 is also active toward substrates with Lys at the corresponding position (4, 13). In the case of Col1a1-2 production in *P. pastoris*, the Met-Gly-Pro-Arg motif was recognized only by Kex2, but the α-factor propeptide could be cleaved by both

Kex2 and Ypsl. The α-factor processing ability of both proteases, together with their divergent sequence specificities, suggests that *kex2* and *ypsl* single-gene disruptants of *P. pastoris* should be evaluated for the expression of heterologous proteins that undergo degradation at basic sites. Whether cleavage of the propeptide by either protease actually occurs probably depends on the particular protein it is fused to, as bulky proteins may result in steric interference. Since the Glu-Ala repeats were not removed from Col1a1-2 in the *kex2* disruptant, this often redundant spacer may need to be omitted from gene constructs with this strain if the native N terminus of the foreign protein is required. In light of the many reports of proteolytic degradation at basic residues in yeasts, *kex2* and *ypsl* disruptants of *P. pastoris* may be very useful for the production of secreted proteins in this powerful expression system.

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